Accomplishments: 1998-present

1998: Mechanisms of Toxicity

A water-soluble toxic activity produced by *Pfiesteria piscidia* has been partially purified in collaboration with North Carolina State University. Reporter gene assays and calcium digital image analysis have been used to investigate the mechanism of action of the putative toxin on calcium signaling pathways in pituitary tumor cells. The putative toxin has also been found to have comparable effects on primary cultures of neurons isolated from memory processing region of the brain (hippocampus) of laboratory rats. *Contact: John Ramsdell*

2000: Potential Toxicity of *Pfiesteria-like* Dinoflagellates Isolated From Florida

Cultures of different species of "Cryptoperidiniopsoid" dinoflagellates were grown under controlled conditions. Each culture was identified via scanning electron microscopy at the Florida Marine Research Institute before shipment to the Marine Biotoxin Program. Each strain was reidentified after mass culture and toxin analysis. Strains were grown in 100 L batches cultures and harvested at late-log growth phase. Production of biological active substances by each culture was examined from both resulting cell mass and spent culture medium. Both cell mass and spent culture medium were passed though a silica column and eluted with an elutropic solvent series. Totals of 5 samples were collected for both the cell mass extract and spent culture medium. Each of the 10 extracts was tested for the possibility of bioactivity using both live assays and cell based assays. Live bioassays included brine shrimp and sheepshead minnows while cell based assay included the GH4C1 cytotoxicity assay. Solvent fractionation yielded several fractions that were active. A non-polar fraction was active on the shrimp bioassay and the sheepshead minnow bioassay. Subsequent structural analysis of this fraction showed this activity in part was due to DEHP, a man-made phthalate ester. This and other fractions are still under pharmacological characterization. A polar fraction was active on the brine shrimp bioassay and the cytotoxicity assay but was inactive on the sheepshead minnow assay. This data provides initial evidence of bioactive substances from cultures of Cryptoperidiniopsoid. Whether this organism produces a toxic substance is presently unknown and will require future pharmacological and chemical investigations. *Contact: Steve Morton*

Preparative Isolation of *Pfiesteria* Toxins From Culture

Methodology for the reproducible isolation of Pfiesteria toxins has been developed and employed for preparative toxin production. This methodology allows toxic fractions to be isolated in a stable form providing a relatively neutral environment free of matrix buffers, enzymes and so forth. Such methodology now enables us to rapidly isolate the toxins from mass culture, quickly removing the toxin(s) from oxidative or reductive environments that would otherwise degrade or destroy the toxin. Purified extracts obtained from this method are being used for toxin characterization both in terms of biological activity as well as molecular characterization using Nuclear Magnetic Resonance and Mass Spectrometry. A lipohpillic toxic fraction has been identified by NMR and GC-MS as bis(diethylhexyl) phthalate, a common plasticizer. The major source for this material has been identified as Instant Ocean. No other non-polar toxin has been observed. The characterization of the polar toxin(s) is well underway. *Contact: Peter Moeller*

Receptor Identified For Putative *Pfiesteria* Toxin Provides Insight Into Effects Of Pfiesteria On Humans And Fish

The pharmacologic activity of a putative toxin (pPfTx) produced by *P. piscicida* has been examined by characterization of the signaling pathways that induce the c-fos luciferase construct in GH_4C_1 rat pituitary cells. A class of purinergic receptors mediates this c-fos pathway with analog selectivity and functional ionic conductances consistent with a purinergic receptor of the P2X7 class. The irreversible P2X7 antagonist, adenosine 5'-triphosphate-2',3'-dialdehyde, was used to demonstrate that the pPfTx requires this pathway for activation. P2X7 receptors are found predominantly on myeloic cells including mature macrophages, mast cells and microglial cells. A role of P2X7 receptors in the action of pPfTx is of interest**,** in consideration of the fact that this toxic dinoflagellate has been reported to cause a range of health impacts in both finfish and humans. The effects linked to *Pfiesteria* toxicity may be related to an inflammatory response, either in macrophages in the periphery or microglia in brain tissue. Implication of P2X7 receptors as a potential target for the bioactive substance produced by toxic *P. piscicida* provides a common basis for the investigation of symptoms that previously have been regarded as unrelated, such as ulcers in menhaden and cognitive dysfunction in humans.

Contact: John Ramsdell

2001: Purification of a Hydrophilic toxin from *Pfiesteria piscicida*

The uncharacterized toxic substances associated with *Pfiesteria piscicida* represent a difficult challenge for health officials. These toxins have been implicated in massive marine mortalities of fish and other wildlife in a number of estuarine systems, and have also been implicated in serious human health related incidents. Without toxin structure and preparative quantities of the toxin itself, very little can be done to mitigate the toxic effects and/or protect human health. This past year we have isolated a polar (water-soluble) toxin from *Pfiesteria piscicida*. This semi-purified material from polar, water-soluble extracts, although not yet analytically pure, is highly cytotoxic and tests positive for the induction of c-fos reporter gene activity, a response mediated by P2X-7 receptor pathways. This information is useful in designing more efficient monitoring techniques as well as providing insights into the toxic substance's mode of action. Mass culture and preparative toxin production will continue.

Contact: Peter Moeller

Toxicogenomics: A Global Approach to Assessing Marine Toxin Exposure and Effects

Toxin exposure almost always causes changes in gene expression, either directly, due to the specific interaction of a toxic agent with its receptor, or indirectly due to the induction of intracellular signaling cascades. Toxicogenomics is the application of DNA arrays to identify a specific pattern of gene expression induced by a particular toxicant. Once a "signature" gene response is identified, this information may be useful for elucidating a toxic mode of action and may potentially yield biomarkers of exposure unique for a particular toxicant or class of toxicants. This year the Marine Biotoxins Program co-organized a workshop on "Toxicogenomics and Nanotechnologies: New Frontiers for Mycotoxins and Phycotoxins" (June 22-23, 2001; Tufts University Bedford, MA) and carried out preliminary studies to determine the suitability of this approach for algal toxin exposure. Changes in gene expression in brains and livers of mice exposed to brevetoxin were studied. Several genes were found to be induced in response to this toxin class. Ongoing studies will determine the dose/response and time course of genetic responses and compare gene induction "signatures" of different algal toxin classes.

Contact: Fran Van Dolah

The Putative Pfiesteria Toxin Tagets Immune Cells to Release Proinflammatory Cytokines

The identification of P2X-7 receptors as a target for the putative *Pfiesteria* toxin led to the unexpected implication of the putative *Pfiesteria* toxin in immune function. P2X-7 receptors are largely restricted to cells of immune origin. This has lead to investigations employing two additional models, macrophages and microglia (macrophages of the brain). This past years work indicates that the putative *Pfiesteria* toxin induces the release of the proinflammatory cytokine interleukin 1b from macrophages. This cytokine is responsible for initiating a cascade of responses leading to inflammation. The hypothesis we are now testing is that the putative *Pfiesteria* toxin disrupts normal immune function. Such a disruption leads to a chronic inflammatory response that is manifest in granulomatous lesions, which is a common response observed in fish exposed to *Pfiesteria*. If this response becomes systemic, it may lead to a septic shock-like effect resulting in massive fish kills.

Contact: John Ramsdell

2002: Toxin Purification from *Pfiesteria* Grown in the Absence of Fish

A new process was developed for producing *Pfiesteria* toxin(s) from cultures grown in the absence of fish. Prior to this development, toxin production by this dinoflagellate appeared to require the presence of fish within the culture. This situation complicated purification methods due to the presence of fish oils, proteins, byproducts, and other non-dinoflagellate debris, which clogged chromatography columns and required additional separation steps in order to obtain clean active samples. The longer time required by the more extensive purification protocol for extracts from fish-cultured algae resulted in unacceptable losses of toxin activity likely due to chemical decomposition. Our new method of culturing *Pfiesteria piscicida* without fish and using *Rhodomonas* sp. as the sole food source has yielded acceptable levels of toxin production and provided cleaner extracts that can be purified with a very rapid three-step procedure. This process has provided NOS scientists with a routine method to produce and maintain enough toxin(s) for structural analysis via 13C, 1H NMR as well as mass spectrometry.

Contact: Steve Morton